Cardiovascular Research (2014) **102**, 118–127
doi:10.1093/cvr/cvt336

Elastin-derived peptides potentiate atherosclerosis through the immune Neu1–PI3Kγ pathway

Stephanie Gayral[1], Roselyne Garnotel[2], Audrey Castaing-Berthou[1], Sebastien Blaise[2], Anne Fougerat[3], Elodie Berge[1], Aurelie Montheil[1], Nicole Malet[1], Matthias P. Wymann[3], Pascal Maurice[2], Laurent Debelle[2], Laurent Martiny[2], Laurent O. Martinez[1], Alexey V. Pshezhetsky[4], Laurent Duca[2†], and Muriel Laffargue[1†*]

[1]INSERM UMR 1048, I2MC, Bât. L3, 1 av Jean-Poulhès, BP 84225, 31432 Toulouse Cedex 4, France; [2]Laboratoire SiRMa, FRE CNRS 3481 MEDyC, URCA, UFR Sciences Exactes et Naturelles, Campus Moulin de la Housse, 51687 Reims Cedex 2, France; [3]Department of Biomedicine, University of Basel Mattenstrasse 28 CH-4058 Basel, Switzerland; and [4]Division of Medical Genetics, Sainte-Justine University Hospital Research Center, 3175 Cote Ste-Catherine, Montréal, PQ, Canada H3T 1C5

Received 2 October 2013; revised 25 November 2013; accepted 9 December 2013; online publish-ahead-of-print 19 December 2013

Time for primary review: 36

Aims Elastin is degraded during vascular ageing and its products, elastin-derived peptides (EP), are present in the human blood circulation. EP binds to the elastin receptor complex (ERC) at the cell surface, composed of elastin-binding protein (EBP), a cathepsin A and a neuraminidase 1. Some *in vitro* functions have clearly been attributed to this binding, but the *in vivo* implications for arterial diseases have never been clearly investigated.

Methods and results Here, we demonstrate that chronic doses of EP injected into mouse models of atherosclerosis increase atherosclerotic plaque size formation. Similar effects were observed following an injection of a VGVAPG peptide, suggesting that the ERC mediates these effects. The absence of phosphoinositide 3-kinase γ (PI3Kγ) in bone marrow-derived cells prevented EP-induced atherosclerosis development, demonstrating that PI3Kγ drive EP-induced arterial lesions. Accordingly, *in vitro* studies showed that PI3Kγ was required for EP-induced monocyte migration and ROS production and that this effect was dependent upon neuraminidase activity. Finally, we showed that degradation of elastic lamellae in LDLR$^{-/-}$ mice fed an atherogenic diet correlated with atherosclerotic plaque formation. At the same time, the absence of the cathepsin A–neuraminidase 1 complex in cells of the haematopoietic lineage abolished atheroma plaque size progression and decreased leucocytes infiltration, clearly demonstrating the role of this complex in atherogenesis and suggesting the involvement of endogenous EP.

Conclusion Altogether, this work identifies EP as an enhancer of atherogenesis and defines the Neuraminidase 1/PI3Kγ signalling pathway as a key mediator of this function *in vitro* and *in vivo*.

Keywords Atherosclerosis • Elastin peptides • Inflammation • Phosphoinositide 3-kinase γ

1. Introduction

Atherosclerosis is a complex, multi-factorial chronic inflammatory disease affecting medium- to large-sized arteries. Recent findings have established a fundamental role for inflammation in mediating the different stages of this disease, revealing an attractive diversity of potential targets that can be exploited in the treatment of this pathology. During early steps of this age-associated inflammatory disease, infiltrated monocytes–macrophages by producing cytokines and reactive oxygen species (ROS) sustain a pro-inflammatory environment in the subendothelial space, resulting in the progression of fatty streak. Owing to this local inflammatory context, the extracellular matrix (ECM) of the arterial wall, mostly composed of the elastin and collagen, is importantly degraded by proteases secreted by infiltrated neutrophil/macrophage. For a long time, the ECM was described as providing support for cells and facilitating the mechanical properties of tissues, but it is now known to also regulate cell phenotype and fate. In the last few years, particular attention has been given to the role of elastin

† Both authors contributed equally to this work.

* Corresponding author. Tel: +33 561325607 (M.L.)/+33 326918939 (L.D.); fax: +33 561325622 (M.L.)/+33 326918366 (L.D.), Email: muriel.laffargue@inserm.fr (M.L.)/laurent.duca@univ-reims.fr (L.D.)

degradation in physiopathological processes as it has been demonstrated that the release of elastases drives the production of elastin-derived peptides (EP) (elastokines) that are proposed to be associated with arterial pathologies.[1] EP are present in the blood of the general population where their serum concentration fluctuates from the ng/mL to the μg/mL range depending on both the procedure used for EP dosage and the type of atherosclerotic disease of the patient.[2–4] Nevertheless, a definitive role for EP in the development of arterial diseases *in vivo* has not yet been demonstrated. *In vitro*, EP control a wide range of biological effects that may play beneficial or deleterious roles on atherosclerosis development. For example, EP are reported to induce monocyte migration and ROS production leading to low-density lipoprotein (LDL) oxidation.[5,6] This suggests that they could aggravate atherosclerosis development *in vivo* and may play a central role in this pathology. In contrast, EP are also reported to mediate endothelial cell nitric oxide production that contributes towards slowing down atheroma plaque formation.[7] In smooth muscle cells, EP are also able to promote cell proliferation, suggesting a potential impact on plaque stabilization *in vivo*.[8] EP is thought to elicit most of these effects by activation of the elastin receptor complex (ERC) which is formed by the association with an elastin-binding protein (EBP) (inactive splicing variant of β-galactosidase protein) and two membrane proteins: the cathepsin A (CathA) or protective protein (PP) and the associated neuraminidase (Neu1). Neu1 was identified as the protein responsible for signal transduction from the ERC leading to the activation of various signalling modules that differ depending on the cell type.[1,9,10] In fibroblasts, ERC activation leads to recruitment and activation of the phosphoinositide 3-kinase γ (PI3Kγ), but the mechanism involved remains unknown.[11] Interestingly, this kinase is highly expressed in haematopoietic cells and plays a key role in inflammatory processes of the arterial wall during atherogenesis.[12,13]

Here, we have evaluated the role of EP in atherosclerosis progression and the possible involvement of PI3Kγ in this process. In this investigation, we used two existing murine models of atherosclerosis to provide direct evidence that EP enhance atheroma plaque size progression and that this effect could be driven by their action on monocytes through the Neu1–PI3Kγ signalling module. We also show that degradation of elastic lamellae in LDLR$^{-/-}$ mice put on an atherogenic diet correlates with atherosclerotic plaque formation. At the same time, the absence of the CathA–Neu1 complex in cells of the haematopoietic lineage abolishes atheroma plaque size progression and leucocyte infiltration, suggesting the involvement of endogenous EP. Altogether our data show a novel function of elastokines in the development of atherogenesis and thus provide new potential targets for the treatment of arterial disease.

2. Methods

2.1 Biological materials

The synthetic peptides VGVAPG and VVGPGA were produced by GeneCust (Luxembourg). The neuraminidase inhibitor 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA) was purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The p110γ antibody was purchased from Santa-Cruz Biotechnology (distributed by Tebu, Le Perray en Yvelines, France). Monoclonal antibodies directed against Akt and phosphorylated Akt (threonine 308), and horse radish peroxydase (HRP)-conjugated anti-rabbit antibodies were purchased from Cell Signaling Technology (distributed by Ozyme, Saint-Quentin en Yvelines, France). The enhanced chemiluminescence (ECL) system was purchased from Amersham Biosciences, Inc. (Orsay, France).

2.2 Mice

PI3K-deficient (PI3Kγ$^{-/-}$) mice were generated from a C57Bl/6J background as previously described.[13–15] CathA$^{S190A\text{-}Neo}$ mice were on a C57Bl/6J background as previously described.[16] As models of *in vivo* atherosclerosis, C57Bl/6J apolipoprotein E-deficient (ApoE$^{-/-}$) mice and C57Bl/6J LDL receptor-deficient (LDLR$^{-/-}$) mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Wild-type (WT), PI3Kγ$^{-/-}$, LDLR$^{-/-}$, and ApoE$^{-/-}$ mice were maintained at the Rangueil Animal Facility of Toulouse (France) and CA$^{S190A\text{-}Neo}$ mice at the animal facilities of the Division of Medical Genetics, Sainte-Justine University Hospital (Montreal, Canada). These mouse models were kept under specific pathogen-free conditions. During experiments, mice were anaesthetized using isoflurane (2%) and were euthanized using cervical dislocation.

All animal procedures were in accordance with institutional guidelines on Animal Experimentation and with a French Ministry of Agriculture license. Moreover, this investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health or the Directive 2010/63/EU of the European Parliament.

2.3 Kappa-elastin preparation

EP were prepared as described previously.[9] Briefly, insoluble elastin was prepared from bovine ligamentum nuchae by hot alkali treatment. Its purity was assessed by comparing its amino acid composition to that predicted by the elastin gene product. Soluble EP were obtained from insoluble elastin by organo-alkaline hydrolysis. This was achieved using 1 M potassium hydroxide in 80% aqueous ethanol. The mixture of EPs obtained is termed kappa-elastin (κE) and exhibits the same biological and physical properties as elastin hydrolysates obtained using elastase taken from leucocytes.

2.4 Bone marrow transplantation

Eight-week-old C57Bl/6J LDLR$^{-/-}$ mice were subjected to medullary aplasia by 9-Gy total-body irradiation. We re-populated mice with i.v. injection of bone marrow (BM) cells isolated from the femurs and tibias of littermate WT or PI3Kγ$^{-/-}$ mice (WT→LDLR$^{-/-}$, PI3Kγ$^{-/-}$→LDLR$^{-/-}$) and littermate WT or CA$^{S190A\text{-}Neo}$ mice (WT→LDLR$^{-/-}$, CathA$^{S190A\text{-}Neo}$→LDLR$^{-/-}$). After 4 weeks of recovery, mice were fed a pro-atherogenic diet (15% fat, 1.25% cholesterol, and 0% cholate) for either an additional 6 weeks for mice reconstituted with PI3Kγ BM, or 12 weeks for mice reconstituted with CathA$^{S190A\text{-}Neo}$ BM. Blood and tissues were collected and analysed as described below. The successful engraftment was confirmed by PCR.

2.5 Characterization of atherosclerotic lesions

The hearts were prepared as described previously[13] and the atherosclerotic lesions were estimated according to Paigen *et al.*[17] Briefly, the heart were washed in phosphate-buffered saline (PBS) and incubated in PBS at 4°C for 12 h. Each heart was frozen on a cryostat mount with optimum cutting temperature (OCT) compound (Tissue-Tek), and stored at −80°C. One hundred sections of 10 μm thickness were prepared from the top of the left ventricle, where the aortic valves were first visible, up to a position in the aorta where the valve cusps were just disappearing from the field. After drying for 2 h, the sections were stained

with Oil Red O and counterstained with Mayer's haematoxylin. After Oil Red O staining, surface lesion areas were measured by computer-assisted image quantification using the Leica QWin software (Leica Microsystems, Wetzlar, Germany). Images were captured with a Sony (Tokyo, Japan) 3CCD video camera.

2.6 Immunohistochemistry

Frozen sections from the aortic root were fixed in acetone/methanol, air-dried, and incubated with 10% of relevant serum for 30 min. The following specific primary antibodies were used: anti-monocyte/macrophage (clone MOMA-2, Serotec, Oxford, UK) and anti-lymphocyte T/CD3 (clone SP7, Zytomed System, Berlin, Germany). Then, sections were incubated with the corresponding secondary biotinylated antibodies (Dako) and visualized with an avidin–biotin–horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories) and the 3-3′ diaminobenzidine (DAB) peroxidase substrate kit (DakoCytomation, Glostrup, Denmark). Countercolouration was performed with Mayer's haematoxylin. Irrelevant IgGs were used as negative controls.

2.7 Analysis of plasmatic cholesterol

Cholesterol levels were measured with a commercial kit: CHOD-PAP (Randox Laboratories, Crumlin, County Antrim, Ireland).

2.8 Murine monocyte ROS production assay

Blood taken from mice was centrifuged (400 *g*) in the presence of a mixture of sodium metrizoate [13.8% (w/v)] and dextran 500 [8.0% (w/v)] (density 1.113 g/L and osmolarity 460 osmol). Monocytes were then purified and washed in Dulbecco's solution (NaCl 137 mmol/L, KCl 2.7 mmol/L, HEPES 30 mmol/L, glucose 10 mmol/L, $CaCl_2$ 1.3 mmol/L, $MgCl_2$ 1.0 mmol/L, pH = 7.4). Cells were counted and their purity was assessed following May–Grunwald–Giemsa colouration. For ROS production, nitroblue tetrazolium (NBT) reduction was used. Cells were stimulated by κE (50 μg/mL), VGVAPG (200 μg/mL), or BSA in the presence or absence of DANA (200 μM), in the presence of NBT. Briefly, 10^4 cells were incubated for 2 h at 37°C in 50 μL DMEM (containing or not the different agonists/inhibitors) in which 5 μL of NBT (1.36 mg/mL) was added. NBT reduction leads to a loss of positive charges and to a consequent decrease in its solubility. Its absorption spectrum is subsequently modified leading from yellow (oxidized form) to blue (reduced form, formazan). The generated insoluble formazan is directly measured using a spectrophotometer (Tecan Infinite F200 Pro, Lyon, France) by absorbance (560 nm).

2.9 Murine monocyte migration assay

Monocyte migration was evaluated using a Boyden chamber containing Dulbecco's solution in the upper compartment and κE (50 μg/mL) or VGVAPG (200 μg/mL) in the presence or in the absence of DANA (200 μM) in the lower compartment. After 4 h incubation, the membrane between the two chambers was stained with crystal violet and cells were counted using a microscope. Ten different fields were counted for each experiment.

2.10 Western blot analysis

Proteins from blood monocytes were dissolved in Laemmli buffer, boiled, separated by SDS–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Immunodetection was achieved using primary antibodies against Akt phosphorylated on Threonine 308 (1/1000), or total Akt (1/1000) overnight at 4°C. Membranes were then incubated with HRP-conjugated secondary antibody (anti-rabbit, 1/1000) for 1 h at room temperature, then immunoreactive proteins were detected with ECL reagents according to the manufacturer's instructions.

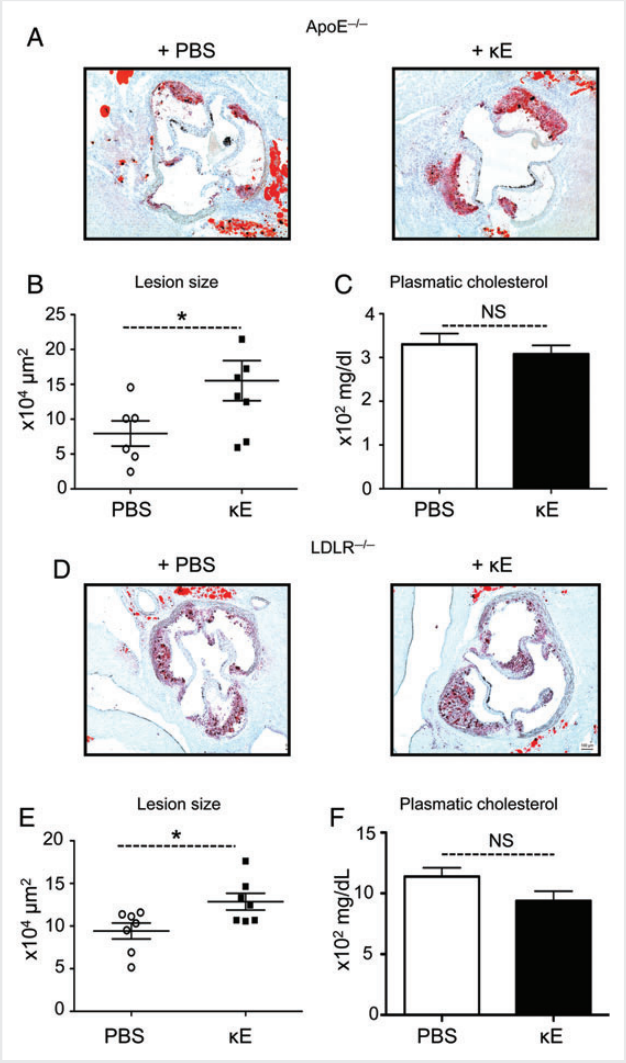

Figure 1 EP increase atherosclerosis development in two atherosclerotic mouse models. (*A–C*) Eleven-week-old ApoE$^{-/-}$ mice were i.v. injected with EP [kappa-elastin (κE)] (5 mg/kg, $n = 7$) or vehicle (PBS, $n = 6$) once a week for 6 weeks, and then spontaneous atherosclerosis was studied. (*D–F*) Eight-week-old LDLR$^{-/-}$ mice were fed an atherogenic diet and i.v. injected with κE (5 mg/kg, $n = 7$) or vehicle (PBS, $n = 7$) once a week for 6 weeks. Representative photomicrographs of Red Oil O-stained fatty streaks (original magnification ×50) (*A* and *D*) and quantitative analysis of atherosclerotic lesion sizes (μm^2) in the aortic roots of indicated mouse models (*B* and *E*) are presented. Total plasma cholesterol levels were measured in each indicated mouse model (*C* and *F*). Data represent means ± SEM. *$P \leq 0.05$ compared with PBS-treated controls; NS, not significantly different from the corresponding control.

2.11 PI3K activity assay

Cells were washed twice in ice-cold PBS containing 50 μM Na_3VO_4, then scraped off into PBS and centrifuged (375 *g*, 10 min, 4°C). Pellets were resuspended and incubated for 15 min at 4°C in immunoprecipitation

lysis buffer [10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Brij 98, 1 mM Na_3VO_4, with added protease inhibitor cocktail]. An insoluble material was removed by centrifugation (20 000 *g*, 20 min, 4°C). Protein concentrations were determined using a bicinchonic acid assay protein assay and equal amounts of proteins were incubated with 10 μg anti-p110γ antibodies for 1 h at 4°C. The antigen–antibody complexes were incubated with protein G-Plus-Sepharose (Santa-Cruz Biotechnology) for 1 h at 4°C, collected by centrifugation, washed three times in immunoprecipitation lysis buffer and then twice with lipid kinase buffer (25 mM HEPES pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 200 μM adenosine). To perform the lipid kinase assay, each pellet was resuspended in 70 μL of lipid kinase buffer supplemented with phosphatidylinositol (PI) and phosphatidylserine (10 mg/mL each), 2.5 μM ATP and 10 μCi [g-^{32}P]-ATP. The reaction was performed for 15 min at 30°C and stopped by the addition of 100 μL 1 M HCl. Phospholipids were extracted with 350 μL chloroform/methanol (1:1, v/v), and the organic phase was washed twice with 200 μL methanol/1 M HCl (1:1, v/v). Organic phases (110 μL) were spotted onto oxalate-treated thin layer chromatography (TLC) plates and lipids were then separated using a chloroform/methanol/acetone/acetic acid/H_2O (40:13:15:12:7, v/v/v/v/v) solvent system. Plates were revealed by autoradiography.

2.12 Elastic fibres breakage analysis

Immediately after sacrifice of mice, thoracic aortas were dissected and then directly frozen in liquid nitrogen. Four-micrometre cryo-sections of OCT-embedded samples were rinsed in PBS for 5 min and conserved with FluorSave TM Reagent (Calbiochem). Autofluorescence of elastin ($\lambda_{excitation}/\lambda_{emission}$: 410/500 nm) was evaluated using a Zeiss microscope (Axio A1).

2.13 Statistical analysis

Comparisons between the different groups were performed using an unpaired *t*-test. Statistical differences considered significant were probability values $P \leq 0.05$ ($^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared with the corresponding control of each experiment). Data are shown as means ± SEM.

3. Results

3.1 EP accelerate atherosclerosis development *in vivo*

To evaluate whether circulating EP play a role in atheroma progression, we used two murine models of atherosclerosis, apolipoprotein E-deficient mice (ApoE$^{-/-}$) and low-density lipoprotein receptor-deficient mice (LDLR$^{-/-}$). We used young ApoE$^{-/-}$ and LDLR$^{-/-}$ mice in which elastic fibre degradation in their arterial wall is very low in order to avoid endogenous EP participation (see Supplementary material online, *Figure 1 A* and *B*). To evaluate the effect of circulating EP in early atherosclerosis, 11-weeks-old ApoE$^{-/-}$ mice were injected with

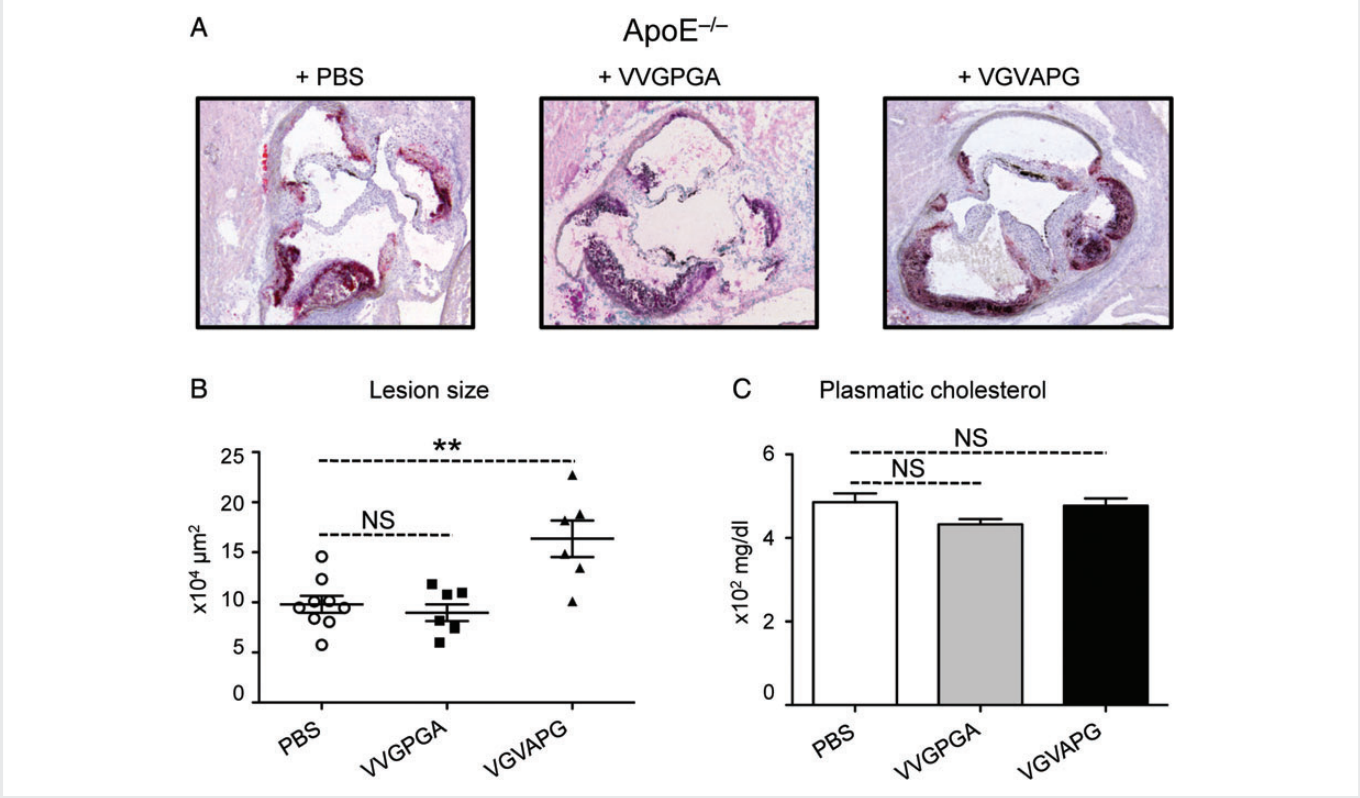

Figure 2 The VGVAPG peptide increases atherosclerosis development. Eleven-week-old ApoE$^{-/-}$ mice were i.v. injected with the synthetic peptides: VGVAPG (20 mg/kg, $n = 9$) or scramble VVGPGA (20 mg/kg, $n = 6$) or vehicle (PBS, $n = 6$) once a week for 6 weeks, then spontaneous atherosclerosis was evaluated. Representative photomicrographs of Red Oil O-stained fatty streaks (original magnification ×50) (*A*) and quantitative analysis of atherosclerotic lesion sizes (μm^2) in the aortic roots of ApoE$^{-/-}$ mice (*B*) are presented. Total plasma cholesterol levels were measured in each treated mouse (*C*). Data represent means ± SEM. $^{**}P \leq 0.01$ compared with PBS-treated controls; NS, not significantly different from the corresponding control.

κE once a week during 6 weeks. κE is an organometallic hydrolysate of elastin that presents the same biological effects as a hydrolysate obtained following digestion of leucocyte elastase. We used a dose of 5 mg/kg κE, a level of EP similar to that found in the general 'atherosclerotic' human population.[3] Analysis of lipid deposition in the aortic root sections of control mice showed fatty streak lesions (on average covering an area of 79 000 μm^2), which were highly potentiated by EP injection (155 000 μm^2) (*Figure 1A* and *B*). Total plasmatic cholesterol levels were not modified by EP injection, suggesting that the increase in the lesion size was not accounted for by changes in plasma cholesterol levels (*Figure 1C*). In parallel, $LDLR^{-/-}$ mice were fed an atherogenic diet for 8 weeks to encourage the development of larger atherosclerotic lesions (120 000 μm^2) (*Figure 1D* and *E*) and presented higher levels of plasmatic cholesterol (*Figure 1F*). Like $ApoE^{-/-}$ mice, these mice also showed a potentiation of atherosclerotic lesion size following EP treatment without any modification of plasmatic cholesterol levels, showing that EP accelerates the development of atherosclerosis in both models. Therefore, these results clearly demonstrate that EP accelerates the initiation of atherosclerosis *in vivo*.

3.2 VGVAPG peptide accelerates atherosclerosis development *in vivo*

It has been established that diverse elastokines containing the XGXXPG repeat hexapeptide can bind to the ERC and that VGVAPG is the optimal ligand for high-affinity binding to this cell surface receptor.[18–20] Therefore, in order to examine whether EP-induced atherosclerosis is specifically dependent on ERC *in vivo*, we used the VGVAPG synthetic peptide. A dose of 20 mg/kg VGVAPG was used since it has been demonstrated that, compared with κE, a four-fold higher concentration of single peptide is necessary to achieve the equivalent physiological stimulation levels of a bioactive EP released following elastin degradation.[20,21] $ApoE^{-/-}$ mice were i.v. injected with VGVAPG peptide or vehicle (PBS) once a week for 6 weeks (*Figure 2*). After red-oil staining of $ApoE^{-/-}$ mice fatty streaks, we observed that VGVAPG synthetic peptides potentiate atherosclerosis progression compared with vehicle-treated mice or to scramble peptide VVGPGA-treated mice (an average lesion size of 163 572 vs. 98 051 μm^2 or 163 572 vs. 89 745 μm^2, respectively). As observed with κE, plasma lipid levels were not affected by VGVAPG treatment or VVGPGA treatment. Thus, these results suggest that specific activation of ERC leads to the potentiation of atherosclerosis.

3.3 Haematopoietic PI3Kγ is required for EP-induced atherosclerosis

Our team has previously described that PI3Kγ is a crucial molecular relay in EP-mediated ERC signalling in fibroblasts.[11] Moreover, we have also provided evidence that loss of PI3Kγ function in the haematopoietic lineage is sufficient to prevent atherosclerosis development.[13] Thus, to evaluate the role of immune PI3Kγ in EP-induced atherosclerosis, we generated a mouse model in which haematopoietic cells exclusively are devoid of PI3Kγ by transplanting BM from PI3Kγ-deficient ($PI3K\gamma^{-/-}$) mice to irradiated $LDLR^{-/-}$-recipient mice ($PI3K\gamma^{-/-} \rightarrow LDLR^{-/-}$). The efficiency of transplantation was established by the detection of <5% of $LDLR^{-/-}$ DNA in BM cells from WT or $PI3K\gamma^{-/-} \rightarrow LDLR^{-/-}$ mice, indicating a chimerism of 95 to 100% of cells (see Supplementary material online, *Figure 2A* and *B*). These mice were injected with κE (5 mg/kg) or vehicle (PBS) once a week as described for *Figure 1*. Analysis of atherosclerotic plaques in aortic roots revealed that EP injections into $WT \rightarrow LDLR^{-/-}$ mice increased atheroma size compared with control mice (average lesion size 48 276 ± 16 469 vs. 79 056 ± 26 435 μm^2) and confirms our results described in *Figure 1* (*Figure 3A* and *B*). Interestingly, injection of EP into $PI3K\gamma^{-/-} \rightarrow LDLR^{-/-}$ mice had no effect on lesion size or plasma cholesterol levels compared with control mice (*Figure 3D, E* and *F*). Thus, these results suggest that PI3Kγ in the haematopoietic lineage regulates atherosclerosis induced by EP signalling.

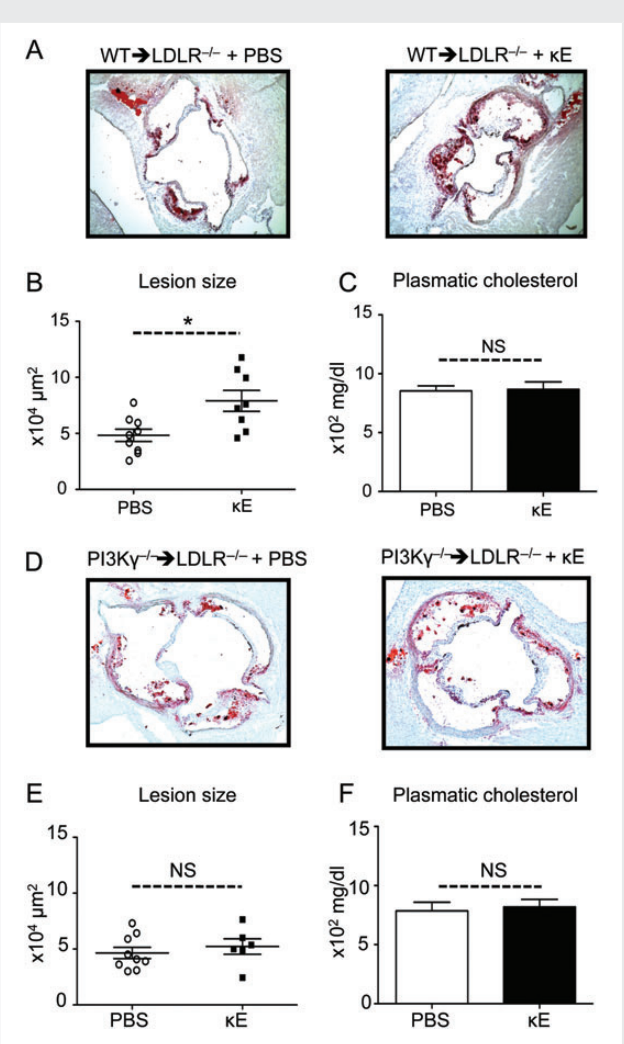

Figure 3 PI3Kγ of the haematopoietic lineage is indispensable for EP signalling-induced atherosclerosis. Eight-week-old irradiated $LDLR^{-/-}$ mice were transplanted with WT BM ($WT \rightarrow LDLR^{-/-}$, $n = 16$) (*A–C*) or with PI3Kγ-deficient littermate ($PI3K\gamma^{-/-}$) BM ($PI3K\gamma^{-/-} \rightarrow LDLR^{-/-}$, $n = 15$) (*D–F*); both were fed a pro-atherogenic diet and i.v. injected with EP [kappa-elastin (κE)] (5 mg/kg) or vehicle (PBS) once a week for 6 weeks. Representative photomicrographs of Red Oil O-stained fatty streaks (*A* and *D*) and quantitative analysis of atherosclerotic lesion sizes (μm^2) in the aortic roots of each transplanted $LDLR^{-/-}$ mouse (*B* and *E*) is shown. Total plasma cholesterol levels were measured in each indicated transplanted mouse (*C* and *F*). Data represent means ± SEM. *$P \leq 0.05$ compared with PBS-treated controls; NS, not significantly different from the corresponding control.

3.4 PI3Kγ and neuraminidase are involved in EP-induced ROS production and migration of monocytes

The biological functions of EP first described in monocytes was their ability to induce the production of ROS and migration, both of which contribute towards atherogenesis.[22–24] We, therefore, investigated the possible role of PI3Kγ and neuraminidase in these processes. Thus, circulating monocytes isolated from PI3Kγ$^{+/+}$ and PI3Kγ$^{-/-}$ mice were stimulated *in vitro* with κE or VGVAPG pre-treated or not with DANA, a specific neuraminidase inhibitor able to block the biological activities of the ERC and elastokine-induced signalling[9,25] (*Figure 4*). EP-induced ROS production (*Figure 4A* and *C*) and migration (*Figure 4B* and *D*) were reduced to the same extent by both the absence of PI3Kγ or following treatment with DANA. Moreover, treatment with DANA had no additional effects on VGVAPG-induced ROS production and migration in PI3Kγ$^{-/-}$ monocytes (data not shown), suggesting that PI3Kγ and neuraminidase are in the same signalling pathway. These results show that neuraminidase and PI3Kγ are key transducers of EP-induced ROS production and migration in monocytes.

3.5 EP control PI3Kγ activity in a neuraminidase-dependant manner in monocytes

To further delineate the signalling cascades involved in EP stimulation of monocytes, we measured the impact of DANA on PI3Kγ activity. We measured PI3Kγ activity in p110γ immunoprecipitates using an *in vitro* PI3K activity assay with PI as a substrate and found that κE treatment increased PI3K activity (*Figure 5A*). To confirm these results, we evaluated the phosphorylation of Akt, a well-known effector of PI3K lipid kinase activity. κE treatment increased Akt phosphorylation on threonine 308 without any changes in total Akt expression (*Figure 5B*), indicating that PI3K activity was increased by EP stimulation. Interestingly, pre-treatment with DANA reduced EP-induced activity back to control levels (*Figure 5A* and *B*), demonstrating that neuraminidase activity is a pre-requisite for EP-induced activation of PI3Kγ in monocytes.

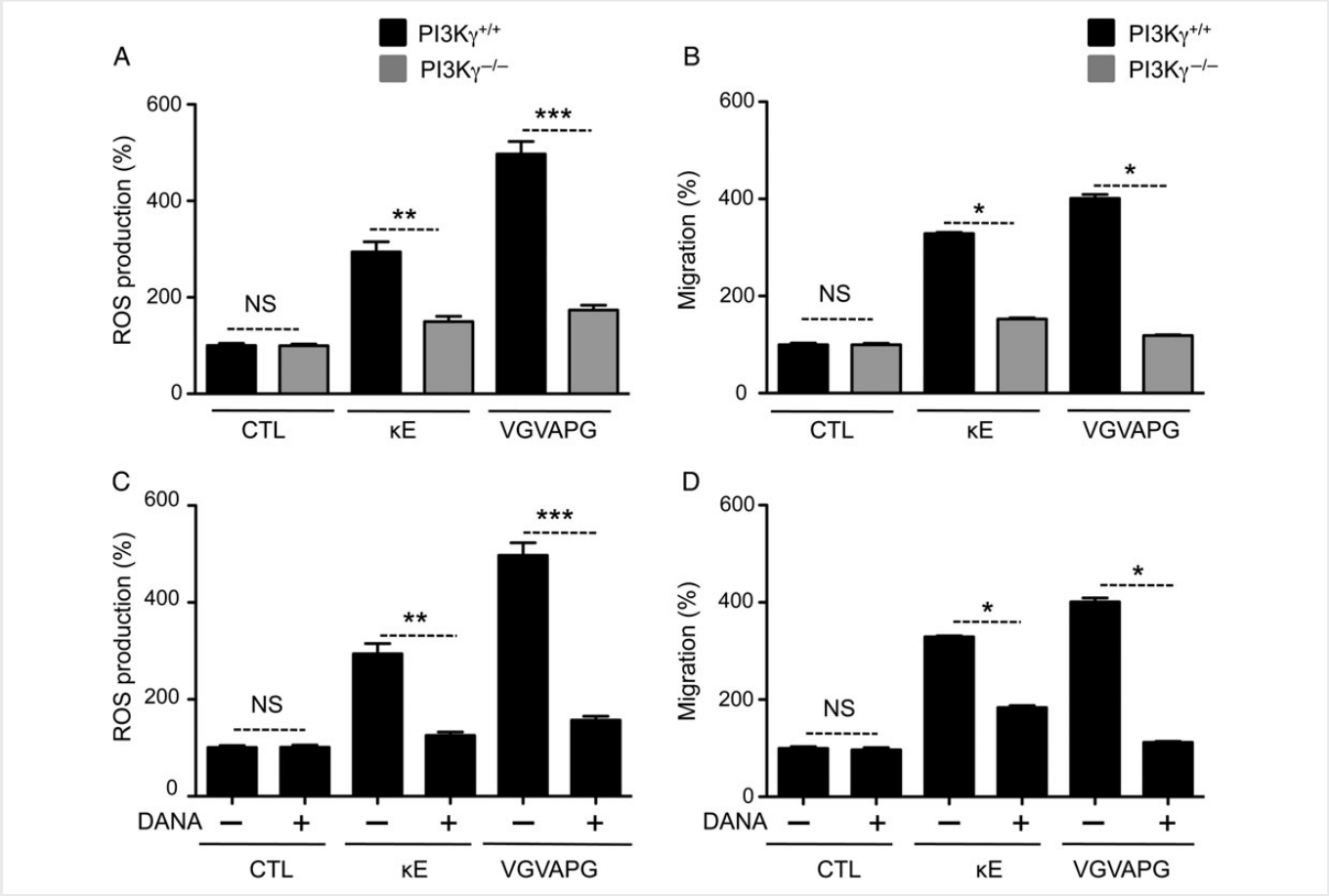

Figure 4 Loss of PI3Kγ expression and Neu inhibition reduces ROS production and migration in monocytes. (*A* and *B*) Monocytes were purified from the blood of PI3Kγ$^{+/+}$ ($n = 5$) or PI3Kγ$^{-/-}$ ($n = 5$) mice and were stimulated with κE (50 μg/mL) or VGVAPG peptide (200 μg/mL) for either 2 h to measure ROS production (*A*) or 4 h to measure migration (*B*) in each indicated condition. Data represent means ± SEM. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$ compared with WT monocytes stimulated with the same agonists. NS, not significantly different from the corresponding control. (*C* and *D*) PI3Kγ$^{+/+}$ monocytes ($n = 5$) were stimulated with κE (50 μg/mL) or VGVAPG peptide (200 μg/mL) in the presence or absence of DANA (200 μM), and then ROS production (*C*) or migration (*D*) were measured in each indicated condition. Data represent means ± SEM. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$ compared with WT monocytes stimulated with the same agonist, NS, not significantly different from the corresponding control.

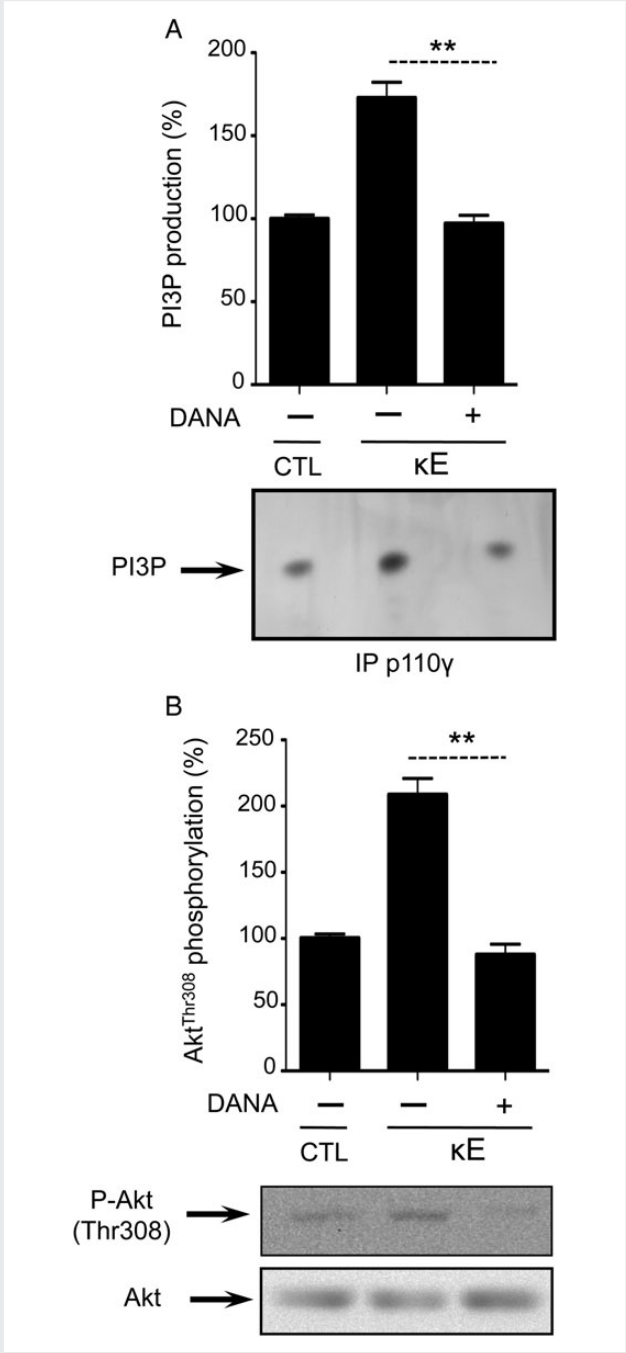

Figure 5 Neuraminidase is responsible for PI3Kγ signalling in monocytes. Blood monocytes were purified from PI3Kγ$^{+/+}$ mice, stimulated with κE (15 min, 50 μg/mL) in the presence or in the absence of DANA. (*A*) An equal amount of protein was subjected to immunoprecipitation using antibodies against the catalytic subunit of PI3Kγ (p110γ). Immunoprecipitates were incubated with a mixture of PI/phosphatidylserine/[γ^{32}P] ATP and PI3P production was measured as an indicator of PI3K activity. Thin layer chromatography (TLC) from representative experiments and densitometry analyses is shown (means ± SEM, $n = 3$). $^{**}P \leq 0.01$ compared with unstimulated cells. (*B*) Lysates from blood monocytes were analysed by western blot analysis using antibodies against phosphorylated Akt (threonine 308) or total Akt. Immunoblots from representative experiments and densitometry analyses are shown (means ± SEM, $n = 3$). $^{**}P \leq 0.01$ compared with unstimulated cells.

3.6 Decreased Neu1 activity in the haematopoietic lineage is sufficient to reduce atherosclerosis in LDLR$^{-/-}$ mice

During the inflammatory response in the progression of atherosclerosis, elastinolytic enzymes are released which cleave insoluble elastin within the arteries to generate endogenous EP.[26] To better identify the dynamics for EP appearance, we observed elastic lamellae degradation in LDLR$^{-/-}$ mice fed an atherogenic diet for 6 or 12 weeks using elastin autofluorescence analysis.[27] No degradation was observed after 6 weeks of atherogenic diet but by 12 weeks elastic lamellae appeared totally degraded, indicating that EP production occurred predominantly after 6 weeks (*Figure 6A*).

To assess the role of Neu1 in atherosclerosis induced by endogenous EP *in vivo*, we generated a mouse model with catalytically defective Neu1 in haematopoietic cells by BM transplant from CathA/Neu1-deficient mice (CathA$^{S190A\text{-}Neo}$ mouse) to irradiated LDLR$^{-/-}$-recipient mice (CathA$^{S190A\text{-}Neo}$ → LDLR$^{-/-}$). These CathA$^{S190A\text{-}Neo}$ mice presented a 90% reduction in Neu1 activity in leucocytes compared with WT cells.[16,28] Transplantation efficiency was verified by the detection of <5% of LDLR$^{-/-}$ DNA in BM cells from CathA$^{S190A\text{-}Neo}$ → LDLR$^{-/-}$ mice, attesting a high percentage of donor BM recovery (see Supplementary material online, *Figure 2A* and *C*). Plasma lipid analysis of CathA$^{S190A\text{-}Neo}$ → LDLR$^{-/-}$ mice fed an atherogenic diet for 12 weeks showed no modification compared with LDLR$^{-/-}$ mice transplanted with WT BM (WT→ LDLR$^{-/-}$) (*Figure 6B*). In contrast analysis of lipid deposition in aortic roots section showed dramatic decrease in the lesion size in CathA$^{S190A\text{-}Neo}$ → LDLR$^{-/-}$ mice compared control mice (*Figure 6C*). Moreover, cellular composition of atherosclerotic lesion showed an important reduction of monocytes and lymphocytes infiltration in the absence of CathA–Neu1 complex (*Figure 6D* and *E*). Altogether, these data indicate that haematopoietic Neu1 expression is indispensable in the formation of atherosclerotic lesions in mice.

4. Discussion

EP are one of the major products of ECM degradation that occurs during inflamm-aging, and now a growing body of evidence suggests that they can also affect adjacent/circulating cells and contribute to the progression of age-associated chronic inflammatory diseases such as atherosclerosis.[1,29] Despite this, an active function for EP in the induction of atheroma plaque progression is yet to be clearly demonstrated. Here, we provide clear evidence that circulating EP are enhancers of atherosclerosis development in two different mouse models of atherosclerosis. Serum concentrations of EP fluctuate from the ng/mL to the μg/mL range depending on the procedure used for EP dosage and the type of atherosclerotic disease of the patients.[2,4,30] Thus, we injected mice with a dose of EP corresponding to the elevated doses found in the circulating blood of the human 'atherosclerotic' population,[2] taking into consideration that 50% of the injected EP was measured in the urine of mice 1 h after injection and that 1 day after injection <10% was detected in the blood.[31] Indeed, it has been described that a circulating dose of 0.5 mg/kg EP can be detected in the blood of general atherosclerotic human population.[2] Therefore, the injection of 5 mg/kg κE leads in 24 h to the same concentration found in humans.

The inductive effect on atherosclerosis development was also observed after injection of a synthetic peptide VGVAPG which is known to bind with high avidity to EBP[19] and transduce signalling path-

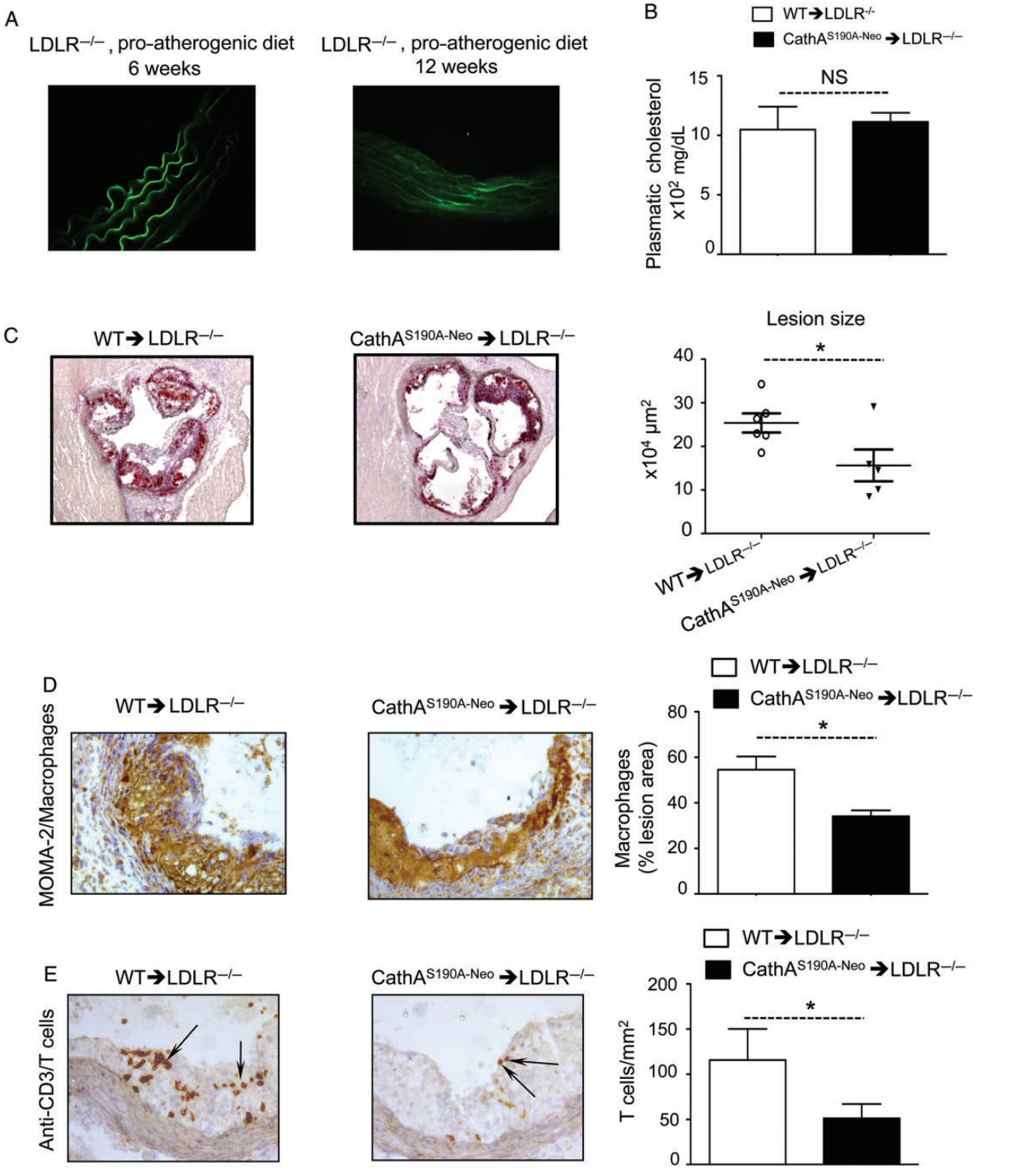

Figure 6 Reduced artherosclerotic lesion size in LDLR$^{-/-}$ mice devoid of Neu1 in immune cells. (*A*) Evaluation of elastic fiber degradation during atherosclerotic ageing in LDLR$^{-/-}$ mice. Autofluorescence of elastin was analysed by epifluorescence from cryo-sections (4 µm) of aorta samples in order to determinate elastic fibre breakage. (*B–E*) Eight-week-old irradiated LDLR$^{-/-}$ mice were transplanted with WT BM (WT→LDLR$^{-/-}$, $n = 6$) or with CA/Neu1-deficient littermate (CathA$^{S190A\text{-}Neo}$) BM (CathA$^{S190A\text{-}Neo}$→LDLR$^{-/-}$, $n = 5$) and fed a pro-atherogenic diet for 12 weeks. Total plasma cholesterol levels were measured in each indicated transplanted mouse (*B*). Atherosclerotic lesion sizes in the aortic roots of each transplanted LDLR$^{-/-}$ mice were analysed and quantified after Red Oil O-staining of the fatty streaks (*C*). Adjacent sections of aortic root with atherosclerotic lesion were stained with anti-monocytes/macrophages (clone MOMA-2) (*D*) and anti-CD3 (T lymphocyte) antibodies (*E*). Data represent means ± SEM. *$P \leq 0.05$ compared with WT→LDLR$^{-/-}$ mice . NS, not significantly different from the corresponding control.

ways through the ERC. Moreover, this effect was not observed in mice treated with the VVGPGA scramble peptide, suggesting that binding of EP to the ERC is involved in atherosclerosis progression in mice. In human cells, ERC has been identified as an heterotrimeric receptor composed of a peripheral 67 kDa EBP, which is a spliced variant of β-galatosidase and specifically binds EP, a 55 kDa PP/CathA and a 61 kDa membrane-bound Neu1 which is the active part of the receptor.[25] Two other EP receptors have been identified to date: the integrin $\alpha_v\beta_3$ and galectin-3,[32] but their *in vivo* relevance is yet to be demonstrated. Moreover, neither is known to have associated sialidase activity like that of Neu1 in the ERC. Consequently, our results can discriminate between the different elastin receptors. As discussed below, we show here that the pro-atherosclerotic effect of EP relies on sialidase activity and a CathA–Neu1 complex, demonstrating a role for the ERC. These results are in par with recently published data implicating the ERC in the induction of type 2 diabetes in a mouse model through Neu1-dependent signalling,[31] confirming a physiological role for the ERC despite the fact that the identity of the EBP in the mouse ERC complex remains to be elucidated. Depending on the cell type, stimulation of the ERC by EP induces activation of various signalling modules.[33] In dermal fibroblasts, we have previously shown that PI3Kγ is essential for activation of the Raf-1/MEK1/2 module following EP stimulation.[11] Interestingly, this isoform of PI3K is highly expressed in immune cells and we and others have shown that it is essential for the inflammatory processes of arterial wall during atherosclerosis development.[13,34,35] We, therefore, hypothesized that this kinase could be the link between ERC and atherosclerosis development. Consistent with these previous data, we conducted transplantation experiments with WT→LDLR$^{-/-}$ and PI3Kγ$^{-/-}$→LDLR$^{-/-}$ mice and found that EP were unable to potentiate atherosclerosis development in the absence of immune PI3Kγ. These results demonstrate for the first time that PI3Kγ is a key signalling protein involved in EP-induced atherosclerosis *in vivo*. Moreover, this result reveals one of the possible mechanisms by which PI3Kγ drives atherosclerosis.

Acting downstream of chemokine receptors, PI3Kγ, plays a major role in modulating the inflammatory functions of immune cells.[36] This kinase is especially indispensable in leucocyte migration in response to chemokines and in the oxidative burst of monocytes in response to fMLP (formyl-methionyl-leucyl-phenylalanine).[14,37] Since *in vitro* studies have demonstrated that EP exert a powerful chemotactic effect on monocytes and stimulate their free-radical production[23,38] we investigated the possibility that PI3Kγ relays signals in both of these biological functions during atherogenesis. Our *in vitro* experiments performed in WT- and PI3Kγ-deficient monocytes provide evidence that neuraminidase activity is essential for PI3Kγ-mediated induction of both functions, but the mechanism by which PI3Kγ is activated remains to be elucidated. One hypothesis is that Neu1-dependent lactosylceramide production described as a central messenger in ERC signalling[10] is linked to the heterotrimeric Gi proteins that are involved in PI3Kγ activation and are known as targets of the ERC.[1] This glycosphingolipid is reported to directly regulate Lyn activation through interaction via its acyl chains. It is thought that the hydrophobic chains of lactosylceramide protrude into the cytoplasmic membrane leaflet and direct van der Waals interactions between itself and the acyl chains of Lyn leading to a conformational modification and activation of Lyn.[39] It is conceivable that the same mechanism applies in the regulation of other acyl chain-anchored proteins such as Gi proteins or other signal tranducers, as has been previously suggested.[40] In addition to their pro-inflammatory effects on monocytes, it has been shown that EPs are able *in vitro* to polarize T cells that express EBP on their surface, shifting them towards the Th1 response.[41] Regarding the importance of this subpopulation of T cells in atherogenesis,[42] we investigated the possible effect of EP in Th1 response *in vivo*. Analysis of the expression of T-bet and TIM-3 expression, both transcription factor specifically activated in Th1 cells, did not show any modification in EP-treated LDLR$^{-/-}$ mice compared with LDLR$^{-/-}$ mice, suggesting that EP were not involved in adaptive immunity during atherogenesis (data not shown). However, it is worth noting that injection of EP represents an artificial model and that the pulse/loading-dose effect of external administration may trigger mechanisms not involved in spontaneous disease. These points bring out the limit of such approach necessitating validation of these data using an endogenous model of EP production.

During the inflammatory response, the release of elastases by leucocytes infiltrating the arterial wall at the site of injury contributes to an increase in the cleavage of insoluble elastin, generating endogenous EP and thus leading to the development of atherosclerosis. We observed a clear degradation of the integrity of elastic lamellae in the arteries of LDLR$^{-/-}$ mice fed an atherogenic diet, suggesting that during atheroma size progression there is a local production of EP in the arterial wall. Previous work conducted *in vitro* showed that the removal of β-Gal or PP/CathA hinders the formation of active Neu1[43] and that Neu1 is an indispensable component of a functional ERC to relay the EP signalling response.[9] To evaluate the effect of endogenous EP on atherosclerosis development, we used a mouse model devoid of CathA/Neu1 catalytic activity in immune cells. Our results demonstrate that a functional immune ERC is indispensable for driving atherosclerosis development, suggesting that Neu1 is essential for mediating the response of endogenous EP in this context. Moreover, decreased infiltrated leucocytes in the absence of Neu1 in haematopoietic lineage suggest that endogenous EP is involved in chemotactism of immune cells reinforcing our *in vitro* data. Nevertheless, it is important to note that the CathA–Neu1 complex is also involved in lysosomal function and other plasma membrane biological processes outside the ERC.[44] Indeed, the ERC derives from a lysosomal degradation complex of β-gal–CathA–Neu1. The generation of a CathA$^{S190A\text{-}Neo}$ mouse leads to the absence of CathA expression and a drastic reduction in Neu1 activity[16] which affects EP signalling since Neu1 transduces its signal, but other biological processes are also affected.

In summary, this work has identified EP as an enhancer of atherogenesis and defines Neu1–PI3Kγ as key mediators of this function, driving the monocyte response to EP. Taken together, our data further illustrate the critical importance of controlling the production of endogenous EP and their signalling downstream of the ERC in the progression of arterial disease.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

We also thank the animal facility and the experimental histopathology platform of the Bio-Medical Research Federative Institute of Toulouse (Genotoul Anexplo Platform IFR150). We thank the Non-Invasive Exploration Service (US006/CREFRE INSERM/UPS) for the use of their irradiator.

Conflict of interest: none declared.

Funding

This work was supported by the Fondation de France (FDF N° 2012-00029502) (M.L.), Midi-Pyrénées region and the National Research Agency (ANR-JCJC n° ANR-12-JSV1-0006) (M.L.) and by the Canadian Diabetes Association (OG-3-10-3128-AP) (A.P.); S.G. was supported by a Foundation Lefoulon-Delalande fellowship and A.F. was supported by a scholarship from the FRQS.

References

1. Maurice P, Blaise S, Gayral S, Debelle L, Laffargue M, Hornebeck W *et al*. Elastin fragmentation and atherosclerosis progression: the elastokine concept. *Trends Cardiovasc Med* 2013;**23**:211–221.
2. Bizbiz L, Alperovitch A, Robert L. Aging of the vascular wall: serum concentration of elastin peptides and elastase inhibitors in relation to cardiovascular risk factors. The EVA study. *Atherosclerosis* 1997;**131**:73–78.
3. Robert L, Robert AM, Jacotot B. Elastin-elastase-atherosclerosis revisited. *Atherosclerosis* 1998;**140**:281–295.
4. Petersen E, Wagberg F, Angquist KA. Serum concentrations of elastin-derived peptides in patients with specific manifestations of atherosclerotic disease. *Eur J Vasc Endovasc Surg* 2002;**24**:440–444.
5. Fulop T Jr, Larbi A, Fortun A, Robert L, Khalil A. Elastin peptides induced oxidation of LDL by phagocytic cells. *Pathol Biol* 2005;**53**:416–423.
6. Robert L, Jacob MP, Frances C, Godeau G, Hornebeck W. Interaction between elastin and elastases and its role in the aging of the arterial wall, skin and other connective tissues. A review. *Mech Ageing Dev* 1984;**28**:155–166.
7. Faury G, Ristori MT, Verdetti J, Jacob MP, Robert L. Effect of elastin peptides on vascular tone. *J Vasc Res* 1995;**32**:112–119.
8. Mochizuki S, Brassart B, Hinek A. Signaling pathways transduced through the elastin receptor facilitate proliferation of arterial smooth muscle cells. *J Biol Chem* 2002;**277**: 44854–44863.
9. Duca L, Blanchevoye C, Cantarelli B, Ghoneim C, Dedieu S, Delacoux F *et al*. The elastin receptor complex transduces signals through the catalytic activity of its Neu-1 subunit. *J Biol Chem* 2007;**282**:12484–12491.
10. Rusciani A, Duca L, Sartelet H, Chatron-Colliet A, Bobichon H, Ploton D *et al*. Elastin peptides signaling relies on neuraminidase-1-dependent lactosylceramide generation. *PloS one* 2010;**5**:e14010.
11. Duca L, Lambert E, Debret R, Rothhut B, Blanchevoye C, Delacoux F *et al*. Elastin peptides activate extracellular signal-regulated kinase 1/2 via a Ras-independent mechanism requiring both p110gamma/Raf-1 and protein kinase A/B-Raf signaling in human skin fibroblasts. *Mol Pharmacol* 2005;**67**:1315–1324.
12. Wymann MP, Zvelebil M, Laffargue M. Phosphoinositide 3-kinase signalling—which way to target? *Trends Pharmacol Sci* 2003;**24**:366–376.
13. Fougerat A, Gayral S, Gourdy P, Schambourg A, Ruckle T, Schwarz MK *et al*. Genetic and pharmacological targeting of phosphoinositide 3-kinase-gamma reduces atherosclerosis and favors plaque stability by modulating inflammatory processes. *Circulation* 2008;**117**: 1310–1317.
14. Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L *et al*. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science* 2000;**287**: 1049–1053.
15. Laffargue M, Calvez R, Finan P, Trifilieff A, Barbier M, Altruda F *et al*. Phosphoinositide 3-kinase gamma is an essential amplifier of mast cell function. *Immunity* 2002;**16**: 441–451.
16. Seyrantepe V, Hinek A, Peng J, Fedjaev M, Ernest S, Kadota Y *et al*. Enzymatic activity of lysosomal carboxypeptidase (cathepsin) A is required for proper elastic fiber formation and inactivation of endothelin-1. *Circulation* 2008;**117**:1973–1981.
17. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* 1987;**68**:231–240.
18. Hinek A, Wrenn DS, Mecham RP, Barondes SH. The elastin receptor: a galactoside-binding protein. *Science* 1988;**239**:1539–1541.
19. Mecham RP, Hinek A, Entwistle R, Wrenn DS, Griffin GL, Senior RM. Elastin binds to a multifunctional 67-kilodalton peripheral membrane protein. *Biochemistry* 1989;**28**: 3716–3722.
20. Blanchevoye C, Floquet N, Scandolera A, Baud S, Maurice P, Bocquet O *et al*. Interaction between the elastin peptide VGVAPG and human elastin binding protein. *J Biol Chem* 2013;**288**:1317–1328.
21. Brassart B, Fuchs P, Huet E, Alix AJ, Wallach J, Tamburro AM *et al*. Conformational dependence of collagenase (matrix metalloproteinase-1) up-regulation by elastin peptides in cultured fibroblasts. *J Biol Chem* 2001;**276**:5222–5227.
22. Senior RM, Griffin GL, Mecham RP. Chemotactic activity of elastin-derived peptides. *J Clin Invest* 1980;**66**:859–862.
23. Fulop T Jr, Jacob MP, Varga Z, Foris G, Leovey A, Robert L. Effect of elastin peptides on human monocytes: Ca^{2+} mobilization, stimulation of respiratory burst and enzyme secretion. *Biochem Biophys Res Commun* 1986;**141**:92–98.
24. Fulop T Jr, Jacob MP, Khalil A, Wallach J, Robert L. Biological effects of elastin peptides. *Pathol Biol* 1998;**46**:497–506.
25. Hinek A, Pshezhetsky AV, von Itzstein M, Starcher B. Lysosomal sialidase (neuraminidase-1) is targeted to the cell surface in a multiprotein complex that facilitates elastic fiber assembly. *J Biol Chem* 2006;**281**:3698–3710.
26. Hornebeck W, Emonard H. The cell-elastin-elastase(s) interacting triade directs elastolysis. *Front Biosci (Landmark Ed)* 2011;**16**:707–722.
27. Aikawa E, Aikawa M, Libby P, Figueiredo JL, Rusanescu G, Iwamoto Y *et al*. Arterial and aortic valve calcification abolished by elastolytic cathepsin S deficiency in chronic renal disease. *Circulation* 2009;**119**:1785–1794.
28. Seyrantepe V, Iannello A, Liang F, Kanshin E, Jayanth P, Samarani S *et al*. Regulation of phagocytosis in macrophages by neuraminidase 1. *J Biol Chem* 2010;**285**:206–215.
29. Fulop T, Khalil A, Larbi A. The role of elastin peptides in modulating the immune response in aging and age-related diseases. *Pathol Biol* 2011;**60**:28–33.
30. Baydanoff S, Nicoloff G, Alexiev C. Age-related changes in the level of circulating elastin-derived peptides in serum from normal and atherosclerotic subjects. *Atherosclerosis* 1987;**66**:163–168.
31. Blaise S, Romier B, Kawecki C, Ghirardi M, Rabenoelina F, Baud S *et al*. Elastin-derived peptides, new regulators of insulin resistance development in mice. *Diabetes* 2013;**62**: 3807–3816.
32. Pocza P, Suli-Vargha H, Darvas Z, Falus A. Locally generated VGVAPG and VAPG elastin-derived peptides amplify melanoma invasion via the galectin-3 receptor. *Int J Cancer* 2008; **122**:1972–1980.
33. Duca L, Floquet N, Alix AJ, Haye B, Debelle L. Elastin as a matrikine. *Crit Rev Oncol Hematol* 2004;**49**:235–244.
34. Chang JD, Sukhova GK, Libby P, Schvartz E, Lichtenstein AH, Field SJ *et al*. Deletion of the phosphoinositide 3-kinase p110gamma gene attenuates murine atherosclerosis. *Proc Natl Acad Sci USA* 2007;**104**:8077–8082.
35. Anzinger JJ, Chang J, Xu Q, Barthwal MK, Bohnacker T, Wymann MP *et al*. Murine bone marrow-derived macrophages differentiated with GM-CSF become foam cells by PI3Kgamma-dependent fluid-phase pinocytosis of native LDL. *J Lipid Res* 2012;**53**:34–42.
36. Fougerat A, Gayral S, Malet N, Briand-Mesange F, Breton-Douillon M, Laffargue M. Phosphoinositide 3-kinases and their role in inflammation: potential clinical targets in atherosclerosis? *Clin Sci (Lond)* 2009;**116**:791–804.
37. Sasaki T, Irie-Sasaki J, Jones RG, Oliveira-dos-Santos AJ, Stanford WL, Bolon B *et al*. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science* 2000;**287**:1040–1046.
38. Hance KA, Tataria M, Ziporin SJ, Lee JK, Thompson RW. Monocyte chemotactic activity in human abdominal aortic aneurysms: role of elastin degradation peptides and the 67-kD cell surface elastin receptor. *J Vasc Surg* 2002;**35**:254–261.
39. Iwabuchi K, Prinetti A, Sonnino S, Mauri L, Kobayashi T, Ishii K *et al*. Involvement of very long fatty acid-containing lactosylceramide in lactosylceramide-mediated superoxide generation and migration in neutrophils. *Glycoconj J* 2008;**25**:357–374.
40. Iwabuchi K, Nagaoka I. Lactosylceramide-enriched glycosphingolipid signaling domain mediates superoxide generation from human neutrophils. *Blood* 2002;**100**:1454–1464.
41. Debret R, Antonicelli F, Theill A, Hornebeck W, Bernard P, Guenounou M *et al*. Elastin-derived peptides induce a T-helper type 1 polarization of human blood lymphocytes. *Arterioscler Thromb Vasc Biol* 2005;**25**:1353–1358.
42. Lahoute C, Herbin O, Mallat Z, Tedgui A. Adaptive immunity in atherosclerosis: mechanisms and future therapeutic targets. *Nat Rev Cardiol* 2011;**8**:348–358.
43. van der Horst GT, Galjart NJ, d'Azzo A, Galjaard H, Verheijen FW. Identification and *in vitro* reconstitution of lysosomal neuraminidase from human placenta. *J Biol Chem* 1989; **264**:1317–1322.
44. Pshezhetsky AV, Hinek A. Where catabolism meets signalling: neuraminidase 1 as a modulator of cell receptors. *Glycoconj J* 2011;**28**:441–452.